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(54) Title: **METHODS AND KITS FOR THE DETECTION OF PRION DISEASES**

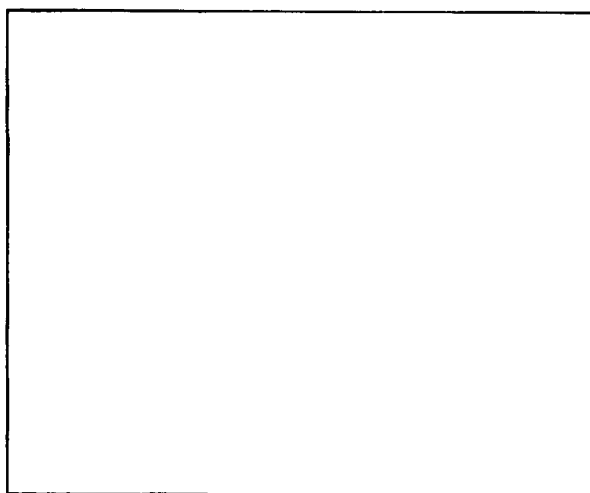
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(57) Abstract: A method and kit for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject comprising: (a) providing a body fluid sample of the subject; (b) qualitatively and/or quantitatively detecting glycosaminoglycans (GAGs) in the sample by suitable means. The presence of GAGs in the sample indicates that the subject carries a prion-associated neurodegenerative disorder, which may be a spongiform encephalopathy such as Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS), Kuru, scrapie and bovine spongiform encephalopathy (BSE).

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METHODS AND KITS FOR THE DETECTION OF PRION DISEASES

Field of the Invention

The present invention relates to methods for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject. More particularly, the invention relates to methods and kits for the diagnosis of prion diseases by detection of GAGs (glycosaminoglycans) in urine samples. The diagnostic methods and kits of the invention are based on the finding that aggregates containing GAGs are formed in urine samples of TSE infected subjects.

Background of the Invention

Prion diseases, also known as TSEs (transmissible spongiform encephalopathies), are a group of fatal neurodegenerative diseases of animals and humans. Among the animal diseases, the most prevalent today is BSE (bovine spongiform encephalopathy) also known as the "Mad Cow Disease". Although less than 100 patients have been diagnosed to date to be BSE-infected, the number of individuals incubating the disease may be millions. Another animal prion disease is scrapie in sheep, which after transmission to rodents constitutes the main experimental prion animal model.

In humans, the most prevalent prion disease is CJD (Creutzfeldt Jakob Disease), which can be manifested either sporadically (about 1 patient per year); genetically (via mutations in the prion protein PrP gene); or in transmissible form, as in the BSE-affected cases. It is a well known experimental fact that the incubation of prion diseases in humans and large animals can last decades.

Prion diseases are believed to be caused by the accumulation in the brain of PrP^{Sc}, an abnormally folded isoform of PrP^C, a GPI anchored protein of unknown function. It has been postulated that prion diseases propagate by

the conversion of PrP^C molecules into protease-resistant and insoluble PrP^{Sc} by an as yet unknown mechanism. The proteinase K (PK) resistant PrP in prion diseases was described by McKinley *et al.* [Cell 35(1):57-62 (1983)]. Immunoblotting of a Proteinase K-digested brain sample infected with a prion disease with an anti-PrP antibody, reveals a characteristic N-terminally truncated PrP protein (the protease resistant core of PrP^{Sc}, denominated PrP 27-30), which is not present in controls or in individuals affected with any other neurological disease.

Diagnosis of prion diseases was based on the presence of this characteristic protease-resistant PrP in brain biopsies, as well as on clinical criteria. Current methods for the conclusive identification of prion diseases include mostly a *post-mortem* analysis of the subject's brain homogenate. Clinical symptoms of the disease can many times be misleading. Evidently, sampling brain tissue from the living subject or patient involves a painful and risky surgical procedure and, moreover, does not give a definite answer since the distribution of PrP^{Sc} in the brain is not uniform. All commercial tests used to date are based on brain presence of protease resistant PrP, for example the Prion-Test of Prionics AG, Switzerland (which company is in charge of most European active surveillance for BSE cases), which is an immunological test for the detection of prions in brain and spinal cord tissue, and is mainly used for BSE and scrapie diagnostics. Since the incubation period in prion diseases is very long (years), it is quite possible that there is a large number of asymptomatic human and animal carriers. There exists therefore a need for developing a simple and readily available pre-clinical and clinical diagnostic non-invasive test for the disease. The need for such an *in vivo* test has been reinforced since the reports of the first cases of variant Creutzfeldt Jakob disease (vCJD) in 1996 [Zeidler, M., et al., Lancet 350(9082):908-10 (1997); Bruce, M. E., et al., Nature 389(6650):498-501 (1997); Ironside, J. W., et al., Histopathology 37(1):1-9 (2000)]. vCJD is a fatal neurodegenerative disease believed to be caused by the consumption of BSE- contaminated meat, and the incubation time between infection to clinical symptoms may be as long as

decades [Bruce, M. E., et al., Nature *ibid.* (1997)]. As opposed to cattle, the incubating individuals will be living for many years, donating blood and in some cases other organs to the non-affected population. Additionally, such test is important for the food industry, and would enable detecting BSE in bovine animals such as cows and sheep, and to prevent marketing of infected meat and dairy products of these animals.

Therefore, a major object of the present invention is the development of a reliable, non-invasive method for diagnosing prion diseases, which will allow the pre-clinical and clinical diagnosis of the disease in humans and in animals.

Since most urine proteins originate from the blood, the present inventors speculated that during the incubation period some PrP^{Sc}, either from the brain or from a peripheral organ, is released into the blood serum in a non-aggregated form, although at low and undetectable concentrations. Due to its protease resistance, PrP^{Sc} is not digested by blood proteases. However, since the MW of PrP is below the cutoff size for filtering through kidney cells (about 40kDA) [Berne, R. M., and Levy, M. N., *Physiology*, 4th Ed (1998)], PrP may subsequently be secreted into the urine and thereby be concentrated, as other proteins, to about 120 folds of its concentration in blood [Kocisko, D. A., et al., Nature 370(6489):471-4 (1994)]. The concentration by the kidney makes it possible to detect PrP^{Sc} in urine more easily than in blood.

The present inventors have previously developed a method for the detection of the protease resistance abnormal isoform of the prion protein, PrP^{Sc}, in a urine sample [WO 02/33420]. The procedure described in this publication is based on the enrichment of the protease-resistant isoform in the urine sample by dialyzing the sample through membrane having a pore range of about 6Kd-8Kd, followed by protease digestion and immunological assay.

The theoretical possibility for diagnosis of prion diseases in a variety of body fluids, such as urine, has been mentioned in several patent documents. EP 0854364, for example, discloses a diagnostic method for neurodegenerative disorders such as Alzheimer's disease and prion diseases. This method is based on concentrating a protein associated with the specific neurodegenerative disease (such as PrP in prion diseases and APP in Alzheimer's disease) in a sample (urine, for example). The concentration is carried out by contacting the sample with a solid, non-buoyant particulate material having free ionic valencies such as calcium phosphate. The detection of only the Alzheimer's disease-associated peptide APP. WO 93/23432 discloses a diagnostic method for prion diseases in different body fluids such as CSF (cerebrospinal fluid) and theoretically, urine. Similarly to EP 0854364, this method is based on concentrating the prion protein by ammonium sulfate precipitation and affinity chromatography. This publication exemplifies CSF as a sample.

Recently, Soto et al. [Saborio, et al., Nature 441:810-813 (2001)] have reported the development of a method for the detection of prion protein by cyclic amplification of protein misfolding (PMCA). This method is based on the rapid conversion of large excess PrP^C into the protease-resistant PrP^{Sc} like form in the presence of minute quantities of PrP^{Sc} template which exist in a positive sample. In this procedure, aggregates formed when PrP^{Sc} is incubated with PrP^C, are disrupted by sonication to generate multiple smaller units for the continued formation of new PrP^{Sc}. According to this method, a tested sample (diluted brain homogenate of a scrapie-affected hamster) was incubated with brain homogenate from healthy hamsters as a source of PrP^C. This mixture was subjected to five cycles of incubation-sonication. The prion protein was then detected using an immunoassay (blot incubated with a PrP^{Sc} specific antibody). Although the authors indicated that this method may be applicable for any body fluid sample, it was only exemplified for brain homogenates of a known PrP^{Sc} infected sample.

The present inventors have recently developed a simple, non-invasive, rapid and protease free method for the detection of PrP^{Sc} aggregates in urine samples of prion infected animals and humans (UPrP^{Sc}) [PCT Application IL2004/000699]. This diagnostic method enhances the aggregation of the abnormal isoform of PrP in a sample, and thereby enables the detection of these aggregates in urine samples obtained from cattle and sheep, without using prion-specific antibodies. This specific enhancement procedure includes the addition of a protein having a beta-sheet structure to the tested sample, preferably IgG light chain (LC), prior to a staining procedure using Congo Red.

Congo Red has been used for many years to identify amyloid aggregates in different tissues. Amyloid aggregates are believed to be composed of proteins and glycosaminoglycans such as heparan sulfate. The proteins comprised within a specific amyloid aggregate may be related to the pathology of a specific disease, such as beta-amyloid for AD and PrP for TSEs. The inventors thus hypothesized that Congo red recognizes amyloid aggregates in urine TSE. Therefore, the possibility of detecting GAGs in urine samples of TSE patients was next examined.

Proteoglycans are widely distributed in animal tissues, occurring predominantly in extracellular matrices, but are also found on cell surfaces. They consist of one or several glycosaminoglycans linked covalently to a protein core. The glycosaminoglycans are composed of repeating disaccharide units, containing carboxylic and/or sulfate ester groups making them acidic by nature [Heinegard, D. and Pausson, M. in Extracellular Matrix Biochemistry (Piez, K.A. and Reddi, A.H. Eds.), 277-328 Elsevier, Amsterdam/New York (1984)].

Several methods have been developed for this purpose, since GAGs in urine are present in several clinical conditions involving renal dysfunction such as diabetes. Interestingly, the only known neurological syndromes in which

GAGS are secreted in urine are the group of recessive genetic diseases known as mucopolysaccharidoses, in which diverse enzymes that degrade GAGs are defected. In one of these neurological genetic diseases, Sanfillipo syndrome, heparan sulfate is known to accumulate in the brain as part of aggregates resembling amyloid deposits present in neurodegenerative diseases.

As will be shown hereunder, three different methods for detection of GAGs performed by the present inventors have clearly indicated the presence of GAGs in urine samples of CJD patients and profound differences between CJD patients and controls were found. Moreover, the inventors found that GAGs are also present in animals very early in the disease incubation, indicating the GAG test can also be used for carriers of the disease and not only in patients exhibiting clinical symptoms. This is very important, since it is known that a number of individuals have contracted vCJD through blood transfusions from healthy individuals who only years later exhibited CJD [Peden, A.H. et al., Lancet. 2004 Aug 7, 364(9433):527-9]

It is therefore one object of the present invention to provide a novel, sensitive and reliable method for the detection of different prion diseases by a non-invasive procedure. The diagnostic method of the invention may be used for the *in-vivo* early diagnosis of ill as well as seemingly healthy but prion-infected individuals.

These and other objects of the invention will become apparent as the description proceeds.

Summary of the Invention

In a first aspect, the present invention relates to a method for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject comprising: (a) providing a body fluid sample of said subject; (b) qualitatively and/or quantitatively detecting glycosaminoglycans (GAGs) in said sample by

suitable means, whereby the presence of GAGs in said sample indicates that said subject carries said neurodegenerative disorder.

According to one embodiment, the method of the invention may further comprise the step of concentrating the sample by a suitable means, before performing the detection.

In another embodiment, suitable means for detecting GAGs in the sample may be any one of optical density spectrophotometry and chromatography methods.

Accordingly, the detection of GAGs in the tested sample may be performed by the following steps: (i) adding to the optionally concentrated sample a binding material capable of binding GAGs and forming a non-soluble precipitate; (ii) applying the precipitate obtained in step (i) onto a solid support; and (iii) detecting a visual signal indicating the presence of GAGs in the tested sample.

In one specific embodiment, suitable means for detecting GAGs in a sample may be any chromatography method such as staining of TLC plates or acrylamide or agarose gels. Accordingly, solid support used by the method of the invention may be any one of TLC plate, acrylamide gel and agarose gel. It should be noted that the visual signal indicating the presence of GAGs, where these methods are used, may be color signal resulting from staining with a specific dye, preferably, Congo red, Azure blue, Alcyan blue, silver and any combination thereof.

The method of the invention is intended for diagnosis of a prion-associated neurodegenerative disorder such as spongiform encephalopathy. More specifically, spongiform encephalopathy may be any one of Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS), Kuru, scrapie and bovine spongiform encephalopathy (BSE).

Still further, the method of the invention is particularly applicable for mammalian subjects such as humans, sheep, goats, bovines, minks, hamsters and felines such as cats.

The body fluid sample used by the method of the invention may be a sample of blood, lymph, milk, urine, faeces, semen, brain extracts, spinal cord fluid (SCF), appendix, spleen and tonsillar tissue extracts. Preferred sample may be a urine sample.

It should be noted that according to a specific embodiment, the tested sample may be concentrated before detection by using centrifugation and precipitation, preferably by using Amicon tubes.

The present invention thus provides a method for the diagnosis of a prion disease in a mammalian subject. According to a particular embodiment, this method comprises: (a) providing a urine sample of said subject; (b) optionally concentrating the sample; (c) detecting glycosaminoglycans (GAGs) in said sample by any one of optical density spectrophotometry and chromatography methods as defined by the invention, whereby the presence of GAGs in said sample indicates that said subject carries said prion disease.

It should be appreciated that diagnosis of prion diseases according to the method of the invention may be performed prior to or after onset of clinical symptoms.

In a further aspect, the present invention relates to a method for detecting the presence of a neurodegenerative disorder-associated aggregates containing GAGs, in a sample of a subject, which method comprises the steps of: (a) concentrating said sample by a suitable means; and (b) qualitatively and/or quantitatively detecting glycosaminoglycans (GAGs) in the sample by suitable means as described by the invention.

In a third aspect, the present invention relates to kit for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject, such kit may comprise: (a) means for obtaining a sample from a tested mammalian subject; (b) means for concentrating the tested sample; (c) means for measuring GAGs in the sample; (d) optionally, suitable buffers; and (e) instructions for carrying out the detection of the presence of aggregates comprising GAGs in the tested sample.

According to one embodiment, the kit of the invention may be designed for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject according to the methods of the invention.

The invention will be further described on hand of the following Figures, which are illustrative only and do not limit the scope of the invention, which is only defined by the appended claims.

Brief Description of the Figures

Fig. 1 Congo red staining of samples obtained from prion infected (BSE) or normal bovine (N), with (+) or without (-) Human IgG Light chain (Lc).

Fig. 2 Congo red staining of samples obtained from healthy individual control (N), AD and from CJD patients. The samples were treated (+) or not-treated (-) with PK prior to separation on 12% SDS PAGE.

Fig. 3 Congo red staining of normal (C), AD and CJD samples which were deproteinized by treatment with Guanidium prior to PK treatment (blot in the left, PK aG). The blot in the right is a control, without Guanidium treatment.

Fig. 4 Urine samples obtained from healthy controls (C), CJD, AD and Sanfillipo (SF) patients were precipitated with CPC and run on a TLC plate. The plate was subsequently stained with Azure Blue.

Fig. 5 Urine samples of three CDJ patients (P), healthy control (C), and heparan sulphate standard (HS) were separated on 1% Agarose gel. The gel was subsequently stained with Alcyan blue.

Fig. 6 Urine samples of three CDJ patients (P), three healthy controls (C), and heparan sulphate standard (HS) were separated on 12% acrylamide gel. The gel was subsequently stained with Alcyan and silver.

Fig. 7A-7B Urine samples of three CDJ patients (P), one suspected patient (S), three healthy controls (C), one AD patient (AD) and Heparan Sulphate standard (HS) were subjected to PK treatment (+/- PK) and separated on two identical 5-20% acrylamide gradient gels.

Fig. 7A One gel was transferred into nitrocellulose membrane that was subsequently incubated with mAb 3F4 (Western blot analysis).

Fig. 7B The second gel was subsequently stained with Alcyan and silver (As).

Detailed Description of the Invention

Extensive evidence has accumulated indicating that several diverse disorders have the same molecular basis, i.e. a change in a protein conformation [Thomas et al., Trends Biochem. Sci. 20:456-459, (1995); Soto, J. Mol. Med. 77:412-418, (1999)]. These protein conformational diseases include -Alzheimer's disease, systemic amyloidosis, Huntington's disease, prion-related disorders (also known as transmissible spongiform encephalopathy), and Amyotrophic Lateral Sclerosis [Soto (1999), supra]. The hallmark event in protein conformational disorders is a change in the secondary and tertiary structure of a normal protein without alteration of the primary structure. The conformationally modified protein may be implicated in the disease by direct

toxic activity, by the lack of biological function of normally-folded protein, or by improper trafficking [Thomas et al., (1995), supra]. In cases where the protein is toxic, it usually self-associates and becomes deposited as amyloid fibrils in diverse organs, inducing tissue damage [Thomas et al., (1995), supra; Kelly, Curr. Opin. Struct. Biol. 6:11-17, (1996); Soto, (1999), supra].

Amyloid is a generic term that describes fibrillar aggregates that have a common structural motif, i.e., the β -pleated sheet conformation [Serpell et al., Cell Mol. Life Sci. 53:887 (1997); Sipe et al., Ann. Rev. Biochem. 61:947-975, (1992)]. These aggregates exhibit specific properties, including the ability to emit a green glow after staining with Congo red, and the capacity to bind the fluorochrome thioflavin S [Sipe, (1992), supra; Ghiso et al., Mol. Neurobiol. 8:49-64, (1994)].

The formation of amyloid is basically a problem of protein folding, whereby a mainly random coil soluble peptide becomes aggregated, adopting a β -pleated sheet conformation [Kelly, (1996), supra; Soto, (1999), supra]. Amyloid formation proceeds by hydrophobic interactions among conformationally altered amyloidic intermediates, which become structurally organized into a β -sheet conformation upon peptide interaction. The hydrophobicity appears to be important to induce interaction of the monomers leading to aggregation, while the β -sheet conformation might determine the ordering of the aggregates in amyloid fibrils.

Prion diseases are associated with the accumulation of a conformational isomer (PrP^{Sc}) of host-derived prion protein (PrP) with an increase in its β -sheet content. According to the protein-only hypothesis, PrP^{Sc} is the principal or sole component of transmissible prions. Although the structure of PrP^C has been determined and has been found to consist predominantly of α -helices, the insolubility of PrP^{Sc}, which is isolated from tissue in a highly aggregated state and which has a high β -sheet content, has precluded high-resolution structural analysis. Various publications [e.g. Hornemann & Glockshuber

PNAS 95:6010-6014 (1998)] describe a β -intermediate which is an unfolding intermediate of mouse PrP and contains predominantly β -sheet elements of secondary structure as opposed to α -helix. Chemical differences have not been detected to distinguish between PrP isoforms and the conversion seems to involve a conformational change whereby the α -helical content of the normal protein diminishes and the amount, of beta-sheet increases. The structural changes are followed by alterations in the biochemical properties: PrP^C is soluble in non-denaturing detergents, PrP^{Sc} is insoluble; PrP^C is readily digested by proteases, while PrP^{Sc} is partially resistant, resulting in the formation of an N-terminally truncated fragment.

Prion diseases are characterized by an extremely long incubation period, followed by a brief and invariably fatal clinical disease. To date, no therapy is available.

As mentioned above, the present inventors have previously established a method for the detection of the abnormal prion protein in urine samples, which was based on specific enrichment procedure including dialysis of the urine sample through membrane having pore range of about 6KD to about 8Kd, followed by protease digestion and immunoassay [WO 02/33420]. The inventors have recently further found [PCT Application IL2004/000699] that PrP^{Sc}, the aberrant isoform and the only known marker for prion diseases, can be identified in the urine of sheep infected with scrapie, cows infected with BSE, as well as in the urine of humans sick with CJD, using a method based on enhancement of aggregate formation in a sample, by addition of a beta-sheet protein such as IgG light chain. Formation of aggregate may then be measured by any known method, such as Congo Red staining followed by dot blot analysis.

The inventors have now surprisingly found, and this is an object of the invention, that GAGs can be detected in urine samples of TSE patients, using well known GAG detecting methods.

The present invention thus provides a rapid, sensitive and specific method for the diagnosis of prion diseases avoiding dialysis, ultracentrifugation, protease digestion and immunological detection steps.

Thus, in a first aspect, the present invention relates to a specific and sensitive method for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject comprising: (a) providing a body fluid sample of said subject; (b) qualitatively and/or quantitatively detecting glycosaminoglycans (GAGs) in said sample by suitable means, whereby the presence of GAGs in said sample indicates that said subject carries said neurodegenerative disorder.

Preferably, the GAGs-containing aggregates which are detected in accordance with the invention, may be non-covalent or non-cross linked aggregates. Covalent aggregates may be formed by disulphide bridges between two cysteine residues present in different proteins and GAGs. Non-covalent aggregates, are not necessarily formed through disulphide bonds, but e.g. through adoption of a high β -structure content. Aggregation typically occurs by nucleation. Nucleation occurs when intermolecular bonds form between polypeptides in a partially or fully denatured state. Important parameters for nucleation, typically include variations in solvents, polypeptide concentration, salt, ligands, temperature and pH. A skilled person will be able to determine suitable conditions for any given GAG.

GAGs can be measured by any technique known in the art. Techniques typically used include optical density spectrophotometry and chromatography methods.

Therefore, according to one embodiment, detection of GAGs may comprise the following steps: (i) adding to a sample, preferably to a concentrated sample, a binding material capable of binding GAGs and forming a non-soluble

precipitate; (ii) applying the precipitate obtained in step (i) onto a solid support; and (iii) detecting a visual signal indicating the presence of GAGs in the tested sample.

As used herein, a "binding material capable of binding GAGs" may include any material such as a protein, peptide, sequence of either, antibody, Congo red, Thioflavin-T, or any species capable of the binding so described. In the case of antibodies, this binding is site-specific. In other cases, it can be non-specific. In a specifically preferred embodiment, the binding material is CPC (cetylpyridinium chloride), which specifically binds and precipitates GAGs.

It should be noted, as shown by Fig. 7, that the GAGs-containing aggregates further comprising proteins associated with neurodegenerative disease. Such proteins, as used herein, mean proteins associated with a prion-associated neurodegenerative disease having sufficient binding capacity to bind to other molecules associated with the neurodegenerative disorder (including like molecules or GAGs), to form fibrils or aggregates characteristic of neurodegenerative disease. Such aggregate-forming proteins typically are characterized by a change in molecule conformation, relative to sequence-homologous, healthy counterparts, allowing them to bind more readily to like or similar molecules. In some cases, such aggregate-forming proteins have the capability to convert proteins from non-aggregate-forming conformation into aggregate-forming conformation.

In a specifically preferred embodiment, suitable means for detecting GAGs in a sample may be any chromatography method such as staining of TLC plates or acrylamide or agarose gels. Accordingly, solid support used by the method of the invention may be any one of TLC plate, acrylamide gel and agarose gel. It should be noted that the visual signal indicating the presence of GAGs, where these methods are used, may be a color signal resulting from staining with a specific dye, preferably, Congo red, Azure blue, Alcyan blue, silver and any combination thereof.

According to another preferred embodiment, the method of the invention is intended for diagnosis of a neurodegenerative disorder, preferably disorder related to amyloidosis or a conformational disease. The term "conformational diseases" refers to that group of disorders arising from propagation of an aberrant conformational transition of an underlying protein, leading to protein aggregation and tissue deposition. Such diseases can also be transmitted by an induced conformational change, propagated from a pathogenic conformer to its normal or non-pathogenic conformer and in this case they are called herein "transmissible conformational disease". Examples of such diseases are spongiform encephalopathies. More specifically, spongiform encephalopathy may be any one of Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS), Kuru and FFI (Fatal familial insomnia) in humans, scrapie in sheep and goats and bovine spongiform encephalopathy (BSE) in cattle, spongiform encephalopathy of exotic ruminants (nyala, gemsbok, Arabian Oryx, eland, kudu, scimitar-horned Oryx, ankole, and bison); feline spongiform encephalopathy (domestic cat, puma, cheetah, ocelot, tiger), CWD (Chronic Wasting Disease) of mule, deer and elk and TME (Transmissible Mink Encephalopathy).

The term "Gerstmann-Strassler-Scheinker Disease" abbreviated as "GSS" refers to a form of inherited human prion disease. The disease occurs from an autosomal dominant disorder. Family members who inherit the mutant gene succumb to GSS.

Still further, the method of the invention is particularly applicable for mammalian subjects such as humans, sheep, goats, bovines, minks, hamsters and felines such as cats.

The body fluid sample used by the method of the invention may be a sample of blood, lymph, milk, urine, faeces, ocular fluids, saliva, semen, brain extracts, spinal cord fluid (SCF), appendix, spleen and tonsillar tissue

extracts, and where necessary can be obtained by biopsy. Preferred sample may be a urine sample.

It should be appreciated that although a preferred sample may be a body fluid sample, the method of the invention may be applicable for any sample.

Therefore, the term "sample" refers to any cell, tissue, or fluid from a biological source, or any other medium that can advantageously be evaluated in accordance with the invention including, but not limited to, a biological sample drawn from a human patient, a sample drawn from an animal, a sample drawn from food designed for human consumption, a sample including food designed for animal consumption such as livestock feed, an organ donation sample, or the like.

It should be noted that according to a specific embodiment, the tested sample is concentrated by centrifugation or precipitation. Preferably, as described by the following Examples, the sample is collected and concentrated using Amicon tubes. It is to be appreciated that any other suitable concentration methods such as dialysis, centrifugation and ethanol precipitation, may be used by the method of the invention.

The present invention thus provides for a method for the diagnosis of a prion disease in a mammalian subject. According to a particular embodiment, this method comprises: (a) providing a urine sample of said subject; (b) concentrating proteins comprised within the sample by using Amicon tube; (c) detecting glycosaminoglycans (GAGs) in the sample by any one of optical density spectrophotometry and chromatography methods as described in the Examples, whereby the presence of GAGs in the sample indicates that said subject carries said prion disease.

It should be appreciated that diagnosis of prion disease according to the method of the invention may be used for diagnosing a prion disease in a

human or animal subject, by obtaining a urine sample of the subject and detecting the presence of GAGs in the urine sample by the detection method of the invention, the presence of the GAGs in the urine of the subject indicating that said subject carries a prion disease. Thus, the invention provides a method for the detection of different prion diseases before or after onset of clinical symptoms.

The diagnostic method of the invention is particularly important for detecting carriers of CJD, for monitoring treatment of CJD patients and for estimating the patient's clinical stage as well as the severity of the disease. It is to be noted that when referring to CJD, all other TSE's are also included. Suspected carriers of pathogenic prion mutations are tested by molecular method for the presence of the mutation, which defines their carrier status. However, and since the age of disease onset can be between 35-85 years or more, there is yet no test to establish at early stages whether the disease is manifesting. Such test could be crucial for early or prophylactic treatment. The detection of carriers of the mutation leading to CJD disease may be used, for example, in genetic counseling.

Additionally, the diagnostic method of the invention is useful in identifying infection of BSE, particularly in individuals that have been exposed to the disease. Identifying human carriers of BSE has importance, *inter alia*, in screening blood samples of human donors for the presence of a prion disease in the donors. Screening can be carried out, for example, by obtaining a urine sample from the donor, detecting the presence of GAGs in the urine sample by the detection method of the invention and ascribing the results of the detection to said blood sample. Such screening would prevent the use of prion-infected blood, thus diminishing risks of blood transfusions.

Additionally, the diagnostic method of the invention, when applied to bovine animals, and also to other domestic animals like sheep and goats or any other animal of interest susceptible to BSE or any other prion disease, may assist

in screening food products originating from the tested animals, like meat and dairy products, and reduce the risk of infection of human consumers.

In a further aspect, the present invention relates to a method for detecting the presence of a prion-associated neurodegenerative disorder-associated aggregate containing GAGs in a sample of a subject, such method comprises the steps of: (a) concentrating the sample, preferably by using Amicon tubes; (b) qualitatively and/or quantitatively detecting glycosaminoglycans (GAGs) in said sample by suitable means.

In a third aspect, the present invention relates to kit for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject, such kit comprising: (a) means for obtaining a sample from a tested mammalian subject; (b) means for concentrating the tested sample; (c) means for measuring GAGs in the sample; (d) optionally, suitable buffers; and (e) instructions for carrying out the detection of the presence of aggregates comprising GAGs in the tested sample.

Where the detection of GAGs in a sample is performed using any known chromatography method, a specific dye such as Congo red, Azure blue, Alcyan blue, silver and any combination thereof may be included in the kit.

Specifically preferred kit according to the invention is particularly applicable for the detection of a prion disease in a mammalian subject, using a urine sample. Such specific kit may preferably comprise means for obtaining a urine sample from the tested subject, means for concentrating the sample, such as Amicon tubes, CPC for precipitating GAGs, solid support for attachment of GAGs in said sample (for example nitrocellulose membrane, TLC plates, acrylamide or agarose gels), further optional buffers and instructions for carrying out the detection of the presence of aggregates comprising GAGs in the tested urine sample.

In general, the kit of the invention is intended for the diagnosis of a prion-associated neurodegenerative disorder such as a spongiform encephalopathy, and is useful in carrying out all of the diagnostic methods of the invention.

It is to be noted that the terms "prion disease" and "prion-associated neurodegenerative disorder" are used herein interchangeably and synonymously.

A number of methods of the art of molecular biology are not detailed herein, as they are well known to the person of skill in the art. Such methods include, for example, detection and analysis of naturally occurring, synthetic and recombinant proteins or peptides and the like. Textbooks describing such methods are e.g., Sambrook *et al.*, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory; ISBN: 0879693096, 1989, Current Protocols in Molecular Biology, by F. M. Ausubel, ISBN: 047150338X, John Wiley & Sons, Inc. 1988, and Short Protocols in Molecular Biology, by F. M. Ausubel *et al.* (eds.) 3rd ed. John Wiley & Sons; ISBN: 0471137812, 1995. These, as well as all other publications cited herein, are incorporated herein in their entirety by reference, including references cited therein. Furthermore, a number of immunological techniques are not in each instance described herein in detail, as they are well known to the person of skill in the art. See e.g., Current Protocols in Immunology, Coligan *et al.* (eds.), John Wiley & Sons, Inc., New York, NY.

Disclosed and described, it is to be understood that this invention is not limited to the particular examples, process steps, and materials disclosed herein as such process steps and materials may vary somewhat. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only and not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

The following examples are representative of techniques employed by the inventors in carrying out aspects of the present invention. It should be appreciated that while these techniques are exemplary of preferred embodiments for the practice of the invention, those of skill in the art, in light of the present disclosure, will recognize that numerous modifications can be made without departing from the intended scope of the invention.

Examples

Experimental procedures

Buffers

- * STE: a. Tris HCl pH7.5, 10mM
- b. NaCl 10mM
- c. EDTA 1 mM pH 8

Membranes for dot blot

- *Nitrocellulose membrane: Schleicher & Schuell, Protran BA83, Cellulose nitrate 0.2µl.

Amicon tubes cat. No. 9031 MINICON B15 were used for concentration of 5ml urine samples.

Protocol for Improved Congo red staining dot-blot

5 ml urine samples obtained from different tested subjects were concentrated using Amicon tubes to a final vol. of 100µl. 50µl of STE + 2% sarcosyl and 0.5 µl of human Light Chain Lambda were added to the concentrated samples. Samples were incubated for 1 h at room temperature or O.N. (over night) at 4°C, followed by centrifugation for 2 min. at 4000 rpm to get a clear sample.

10µl of the sarcosyl solution were added to 10 µl of sample, mixed by pipeting, and then 2 µl of Congo red fresh solution were added to a final concentration of 200 µg/ ml (stock solution: 2 mg/ ml DDW) and incubated for 1h or more. 2 µl of the mixture were applied to a nitrocellulose membrane and the membrane was dried and subjected to washes as follows:.

- 1 min. with DDW;
 - 1 min. with 50% MeOH in DDW;
 - 1 min. with 70% MeOH;
 - 1 min with 80% MeOH;
 - Incubation in 90% MeOH until the negative control disappears.
- Sometimes it is required to go up to 94% methanol.

Determination of total urinary GAG excretion

The total urine GAG content was determined by a CPC (cetylpyridinium chloride) GAG method [Peonock, C.A., J. Clin. Pathol. 29:111 (1976); Di Ferrante, N. Anal. Biochem. 21:98 (1967)]. Under controlled pH and electrolyte concentration conditions, CPC reacts with GAGs to form an insoluble precipitate. In the presence of a citrate buffer at pH 4.8 this precipitate is sufficiently stabilized and dispersed to measure the absorbance -of 680 nm in a spectrophotometer (Mod Du 650, Beckman Industries, Fullerton, CA, USA). Ch-4-S was used as a reference standard. GAG excretion was expressed as a GAGs/creatinine ratio (mg GAG excreted per gram of creatinine) [Peonock, (1976) *ibid.* ; Di Ferrante, (1967) *ibid.*].

Isolation of GAGs

One milliliter of CPC reagent was added to an aliquot of urine. After overnight CPC precipitation at 4°C, the harvested precipitate was mixed with 2-3 mL of potassium acetate in ethanol, and the precipitates were washed with an aliquot of alcohol reagent, harvested by centrifugation, washed once with a few milliliters of diethyl ether, air dried, and taken up in deionized water [Peonock, (1976) *ibid.*].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of glycosaminoglycans was performed on a PhastSystem (pharmacia/LKB) using 12% or 5-20% gels. SDS-PAGE was carried out under non-reducing conditions following the protocol described by the manufacturer. Samples were diluted in sample buffer (final composition of 7.8 mM Tris/HCl, 6% (W/V) urea, 0.875% SDS (W/V), 2.5% glycerol (V/V), 0.625 mM EDTA, and 0.00025% bromophenol blue, pH 8.9) and heated at 100°C for 4 min.

Alcyan blue/neutral silver stain

Immediately after electrophoresis, the gels were placed in the development chamber of the PhastSystem and stained according to Table 1. The method is a combination of Alcyan blue staining (steps 1-7) and a neutral silver staining protocol (steps 8-20) from PhastGel Silver Kit, which is a modification of a method by Heukeshoven and Dernick [Electrophoresis 6:108-112 (1985)].

When combining primary Alcyan blue staining with a silver stain it is crucial to remove unbound Alcyan blue from the gel, which will otherwise precipitate silver ions and produce excessive background staining. This is done by washing thoroughly (steps 5-9) after staining with Alcyan blue. Furthermore very careful initial washing (steps 1-3) is necessary to avoid precipitation of Alcyan blue by SDS.

As for the silver stains the development steps (step 18 and 19) have to be adjusted to the desired sensitivity and background staining. This is most conveniently accomplished by initially performing these steps in Petri dishes. Buffer strips and gels should be handled with gloves or forceps. Water of highest purity should be used the silver nitrate and developer solutions should be prepared fresh on the day of use. All other reagents are stable for several days.

Table 1

Method for Staining with Alcyan Blue and Neutral Silver of Polyacrylamide Gels after SDS Electrophoresis.

Step	Solution	Preparation	Min	°C
1	Fix/wash I	25% EtOH ^a (v/v). 10%	5	50
2	-	HAc ^b (v/v) in water	10	50
3	-		15	50
4	Stain I	0.125% Alcyan blue (w/v) in fix wash I	15	50
5	Fix/wash I	25% EtOH (v/v), 10%	1	50
6	-	HAc (v/v) in water	4	50
7	-		5	50
8	Wash II	10% EtOH (v/v), 5% HAc	2	50
9	-	(v/v) in water	4	50
10	Sensitizer	5% (v/v) Glutardialdehyde	6	50
11	Wash II	10% EtOH (v/v), 5% HAc	2	50
12	-	(v/v) in water	5	50
13	Wash III	Water	2	50
14	-		2	50
15	Stain II	0.4% (w/v) silver nitrate	6.5	40
16	Wash III	Water	0.5	30
17	-		0.5	30
18	Developer	2.5% (w/v) Sodium	0.5	25
19	-	carbonate, 0.013% (v/v) formaldehyde	4-8	25
20	Stop	10% HAc (v/v), 10% glycerol	5	25

a. EtOH: ethanol.

b. HAc: Acetic acid.

Polysaccharide analysis on TLC

GAGs analysis on TLC plates was performed as previously described [Humbel, R. et al., Helvetica Paediatrica Acta, 6:648 (1969)]. Briefly, the following reagents were used:

- 1) CPC – 5% in IM citrate buffer ph-6
- 2) EtoH – 95% saturated with NaCl
- 3) Silica Gel H (G²) plates

- 4) W-propanol
- 5) Ammonium hydroxide – approximately 58%
should be fresh not more than 8 openings of each bottle
- 6) Stain: 125 mg Azure Blue in 150 ml methanol – add 50 ml acetone and
50 ml 2% acetic acid
- 7) 7% acetic acid

Method

- 1) Centrifuge 5 ml urine and transfer supernatant to a small tube –
stopped;
- 2) add 0.1ml CPC solution to the tube, mix and refrigerate overnight;
- 3) spin at greater speed than 2500 rpm, discard supernatant;
- 4) Suspend by pipeting in 2.5 ml salt, saturated EtOH spin for 20' ;
- 5) Discard supernatant, dissolve precipitate in 0.1ml 0.025M NaOH;
- 6) Apply 40ml in 2 cm strip on TLC, no heating;
- 7) Chromatography: in propanol: ammonium hydroxide: water 40: 60: 5 in
cold room (about 6hrs). Dry at room temp;
- 8) Immerse plate in stain 5-10 minutes; and
- 9) Destain in 7% acetic acid (10-20').

Example 1

CR staining detects amyloid aggregates in CJD urine samples

In a search for improved, sensitive and specific non-invasive diagnostic methods for the detection of the prion protein PrP in urine samples of different subjects, the inventors developed a protocol for TSE urine testing, based on Congo Red (CR) staining of urine prion aggregates. In this test, urine from CJD patients and scrapie-infected hamsters could be stained with CR following concentration of the urine sample, and identified as positive by a dot blot protocol. The inventors further found that addition of protein having a beta-sheet conformation to a sample containing the prion protein may enhance aggregation and thus increase the signal.

Based on these findings, the inventors have developed a protocol for a sensitive and specific detection of PrP protein in prion-infected samples. This protocol is based on the addition of external protein having beta-sheet structure, preferably, IgG light chain (LC), to the concentrated urine samples. Following incubation for 2 to 20h, Congo Red is added and the samples are subjected to a dot blot assay as indicated in the experimental procedures.

As shown by Fig. 1, urine samples obtained from infected bovine (BSE) were stained with CR only in the presence of human IgG LC. No signal was shown when normal control samples were used.

The inventors next checked whether the proteins recognized by Congo red (CR) are protease-resistant. Thus, urine samples obtained from a CJD patient, a patient suffering from Alzheimer Disease (AD) and a healthy individual control were treated with PK (Proteinase K) and were separated on 12% SDS PAGE prior to staining with CR. As shown by Fig. 2, a variety of fragments were recognized by CR in AD and CJD untreated samples. However, only samples obtained from CJD patients were recognized by CR after PK treatment. Furthermore, the staining pattern of the PK-treated CJD sample further indicates that PrP is not the sole protease resistant protein in prion disease urine. The inventors therefore hypothesized that prion urine comprises amyloid "seed" which forms a center for aggregation of variety of protein which leads to formation of amyloid accumulation.

The inventors next checked whether CR binds any components other than protease resistant proteins in urine samples of CJD patients. Therefore, urine samples obtained from two CJD patients, one AD patient and two healthy individuals (control), were subjected to Guanidium treatment, which leads to deproteinization by denaturation of proteins, prior to treatment with PK. The samples were then subjected to dot-blot and stained with CR. Fig. 3

shows that only CJD samples were recognized by CD, after deproteination followed by PK treatment. It should be noted that Guanidium treatment significantly reduced the CR signal, indicating that CR binds to amyloid aggregates composed of proteins and some other components.

Example 2

Diagnosis of CJD by the detection of GAGs

Since amyloid aggregates are known as being composed of proteins and glycosaminoglycans (GAGs), the inventors next examined the presence of GAGs in urine samples obtained from CJD patients. Several methods have been previously developed for this purpose since GAGs in urine are present in several clinical conditions involving renal dysfunction such as diabetes and in a group of recessive genetic diseases called mucopolysaccharidoses. Thus, urine samples obtained from two CJD patients, one AD, three healthy individuals (control) and from a patient who suffers from Sanfillipo syndrome (positive control), were subjected to CPC precipitation followed by separation on TLC plates and staining with Azure Blue as indicated in the Experimental procedures. It should be noted that Sanfillipo syndrome is a mucopolysaccharidose disorder characterized by accumulation of heparan sulfate in the brain as part of amyloid-like aggregates. As shown by Fig. 4, the presence of GAGs in urine samples of CJD patients, as well as in the Sanfillipo sample, was clearly detected. No signal was detected in control and AD samples. These results were supported with different methods for GAG detection, such as CPC precipitation, followed by any one of 1% agarose gel and Alcyan blue staining (Fig. 5), 12% acrylamide and Alcyan/silver staining (Fig. 6) and 5-20% Acrylamide gradient with silver staining (Fig. 7B). Fig. 7A further shows immunoblot analysis performed in the same 5-20% gradient gel, using the 3F4 antibody indicating the presence of PrP in urine samples of CJD patients. When these samples were treated with PK and stained with silver (Fig. 7B), the GAGs signal was lower, indicating that heparan sulphate in CJD urine is bound to specific proteins, presumably, PrP.

Quantitative analysis of GAGs in urine samples of CJD patients, control samples and patients after stroke, further supported the results obtained using the different chromatography methods. As shown by Table 2, urine samples of all five CJD patients contain GAGs.

Table 2

Measurement of GAG concentrations in urine

mg GAGs /gr creatinine	Sample
-	control
-	control
-	control
-	control
-	stroke
290	stroke
438	CJD
4587	CJD
832	CJD
268	CJD
190	CJD

Claims

1. A method for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject comprising:
 - (a) providing a body fluid sample of said subject; and
 - (b) qualitatively and/or quantitatively detecting glycosaminoglycans (GAGs) in said sample by suitable means, whereby the presence of GAGs in said sample indicates that said subject carries said neurodegenerative disorder.
2. The method according to claim 1, further comprising the step of concentrating said sample by a suitable means.
3. The method according to any one of claims 1 and 2, wherein suitable means for detecting GAGs in a sample is any one of optical density spectrophotometry and chromatography methods.
4. The method according to claim 3, wherein the detection of GAGs in said step (c) comprises the following steps:
 - (i) adding to the concentrated sample a binding material capable of binding GAGs and forming a non-soluble precipitate;
 - (ii) applying the precipitate obtained in step (i) onto a solid support; and
 - (iii) detecting a visual signal which indicates the presence of GAGs in said tested sample.
5. The method according to claim 4, wherein said binding material is CPC (cetylpyridinium chloride).
6. The method according to claim 5, wherein said suitable means for detecting GAGs in a sample is a chromatography method.

7. The method according to claim 6, wherein said solid support is any one of TLC plate, acrylamide gel and agarose gel.
8. The method according to claim 7, wherein said visual signal is a colored signal resulting from staining with a specific dye.
9. The method according to claim 8, wherein said dye is any one of Congo red, Azure blue, Alcyan blue, silver and any combination thereof.
10. The method according to any of the preceding claims, wherein said neurodegenerative disorder is a spongiform encephalopathy.
11. The method according to claim 10, wherein said spongiform encephalopathy is any one of Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS), Kuru, scrapie and bovine spongiform encephalopathy (BSE).
12. The method according to claim 11, wherein said mammalian subject is selected from the group consisting of humans, sheep, goats, bovines, minks, hamsters and cats.
13. The method according to claim 1, wherein said body fluid sample is selected from the group consisting of: blood, lymph, milk, urine, faeces, semen, brain extracts, spinal cord fluid (SCF), appendix, spleen and tonsillar tissue extracts.
14. The method according to claim 13, wherein said body fluid sample is a urine sample.
15. The method according to claim 1, wherein concentration of said sample is performed by centrifugation and precipitation.

16. A method for the diagnosis of a prion disease in a mammalian subject comprising:
 - (a) providing a urine sample of said subject;
 - (b) concentrating said sample; and
 - (c) detecting glycosaminoglycans (GAGs) in said sample by any one of optical density spectrophotometry and chromatography methods as defined in any one of claims 3 to 9, whereby the presence of GAGs in said sample indicates that said subject carries said prion disease.
17. The method according to claim 16, wherein diagnosis of said prion disease is performed prior to or after onset of clinical symptoms.
18. A method for detecting the presence of a prion-associated neurodegenerative disorder-associated aggregates containing proteins associated with said disorder in a body fluid sample of a subject, said method comprising the steps of:
 - (a) concentrating said sample by a suitable means; and
 - (b) qualitatively and/or quantitatively detecting glycosaminoglycans (GAGs) in said sample by suitable means.
19. The method according to claim 18, wherein said body fluid sample is selected from the group consisting of: blood, lymph, milk, urine, faeces, semen, brain extracts, spinal cord fluid (SCF), appendix, spleen and tonsillar tissue extracts.
20. The method according to claim 19, wherein said body fluid sample is a urine sample.
21. A kit for the diagnosis of a prion-associated neurodegenerative disorder in a mammalian subject, comprising:
 - (a) means for obtaining a sample from a tested mammalian subject;

- (b) means for concentrating said sample;
 - (c) means for measuring GAGs in said sample;
 - (d) optionally, suitable buffers; and
 - (f) instructions for carrying out the detection of the presence of GAGs in said sample.
22. The kit according to claim 21, wherein said neurodegenerative disorder is a spongiform encephalopathy.
23. The kit according to claim 22, wherein said Spongiform encephalopathy is any one of Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker Syndrome (GSS), Kuru, Scrapie and Bovine Spongiform Encephalopathy (BSE).
24. The kit according to claim 23, wherein said mammalian subject is selected from the group consisting of: humans, sheep, goats, bovines, minks, hamsters and cats.
25. The kit according to claim 24, wherein said body fluid sample is selected from the group consisting of: blood, lymph, milk, urine, faeces, semen, brain extracts, spinal cord fluid (SCF), appendix, spleen and tonsillar tissue extracts.
26. The kit according to claim 25, wherein said body fluid sample is a urine sample.

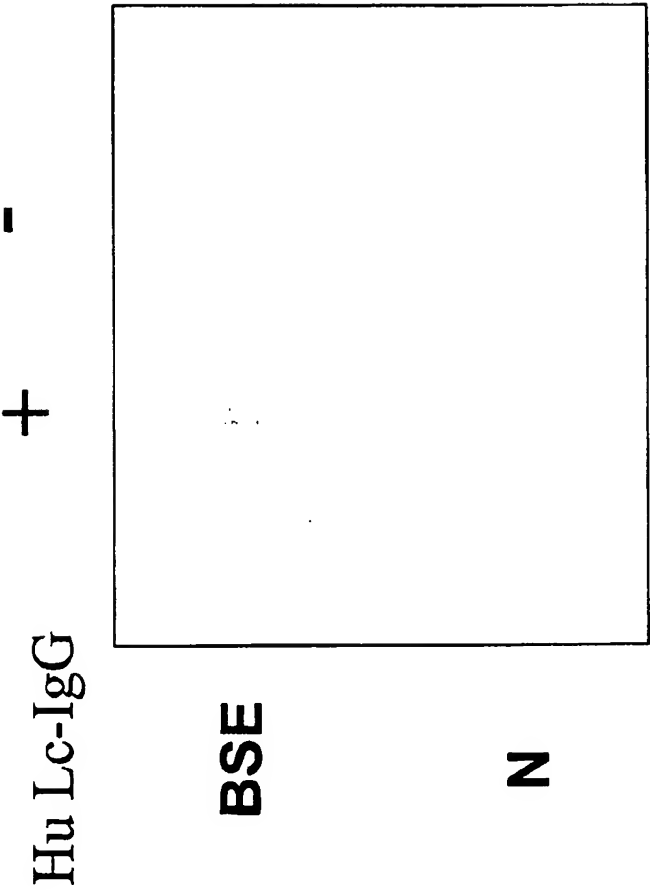


Fig. 1

2/6

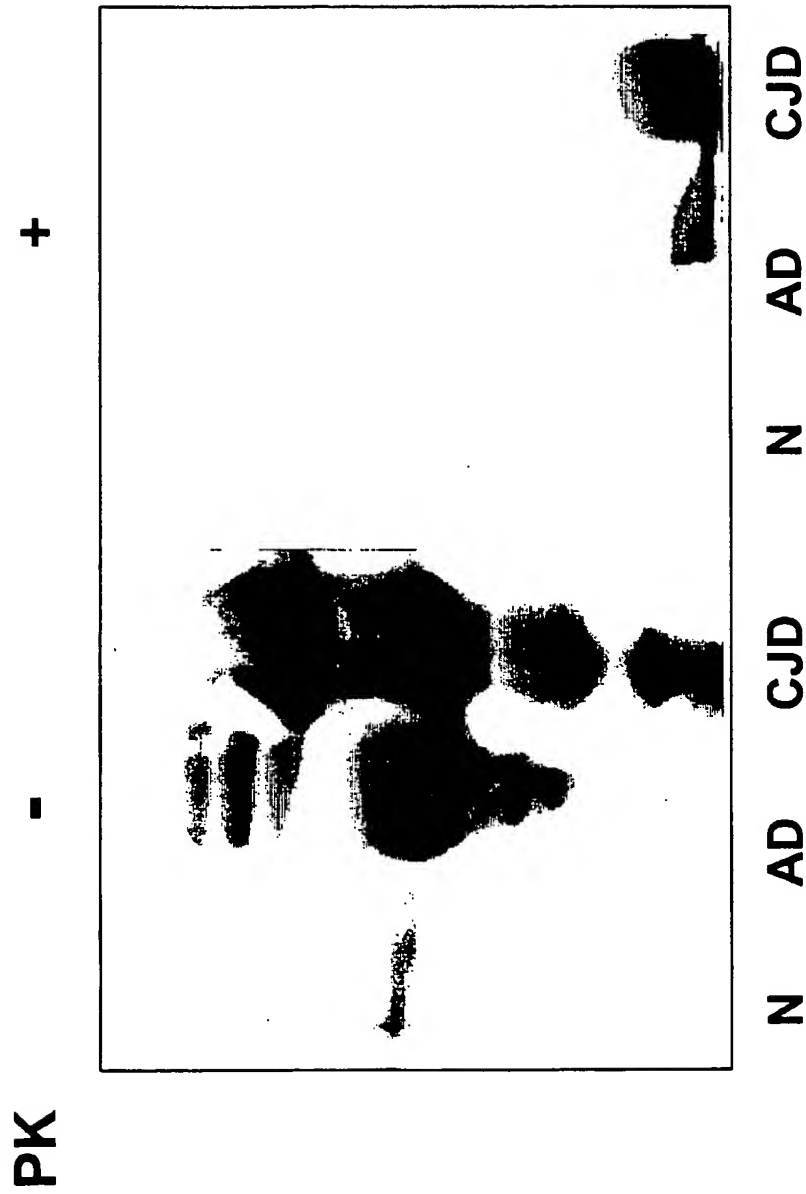


Fig. 2

3/6



CJD CJD C C AD

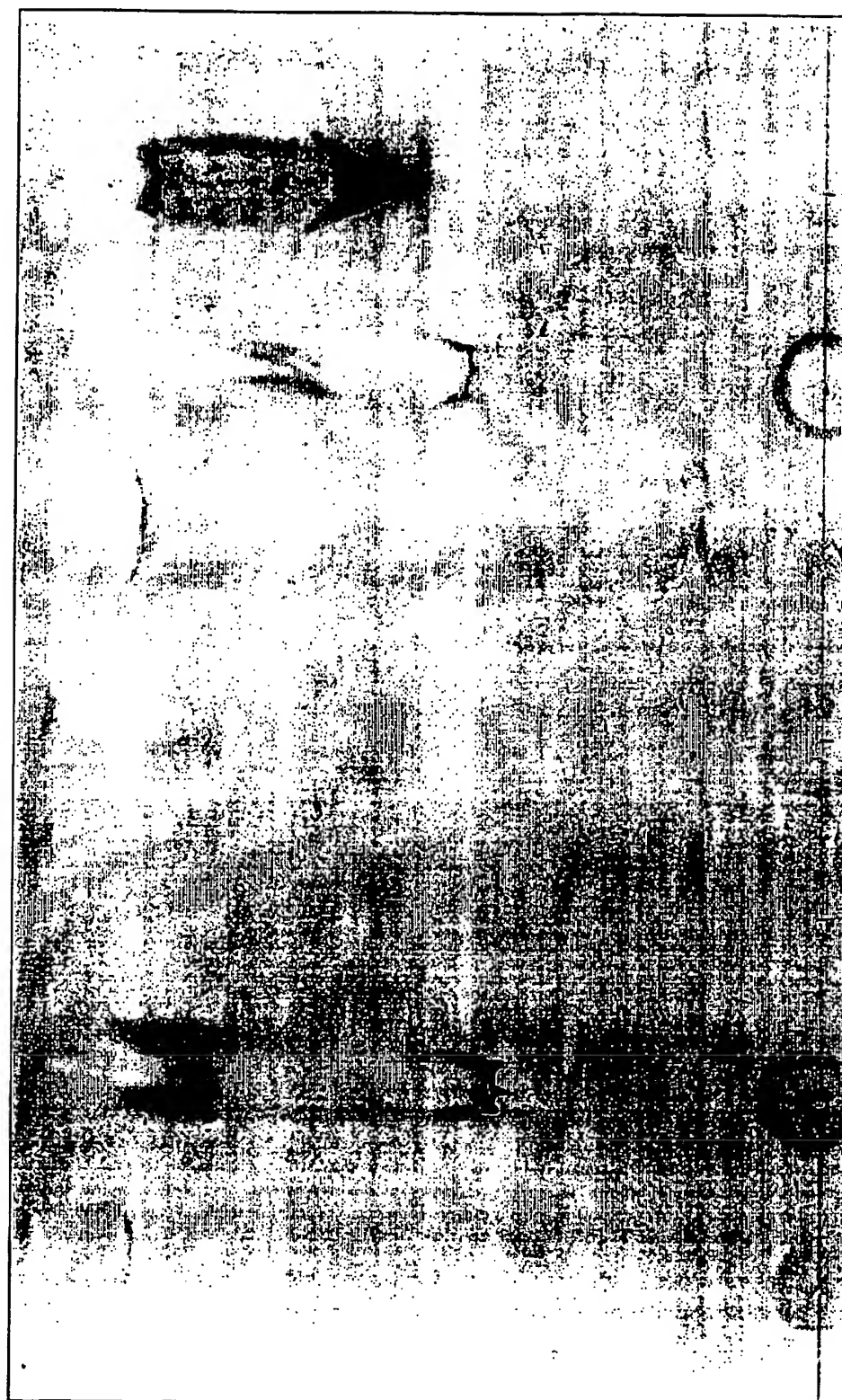
PK



CJD CJD C C AD

PK a.G.

Fig. 3



C CJD C AD C C CJD C CJD C SF

Fig. 4

5/6

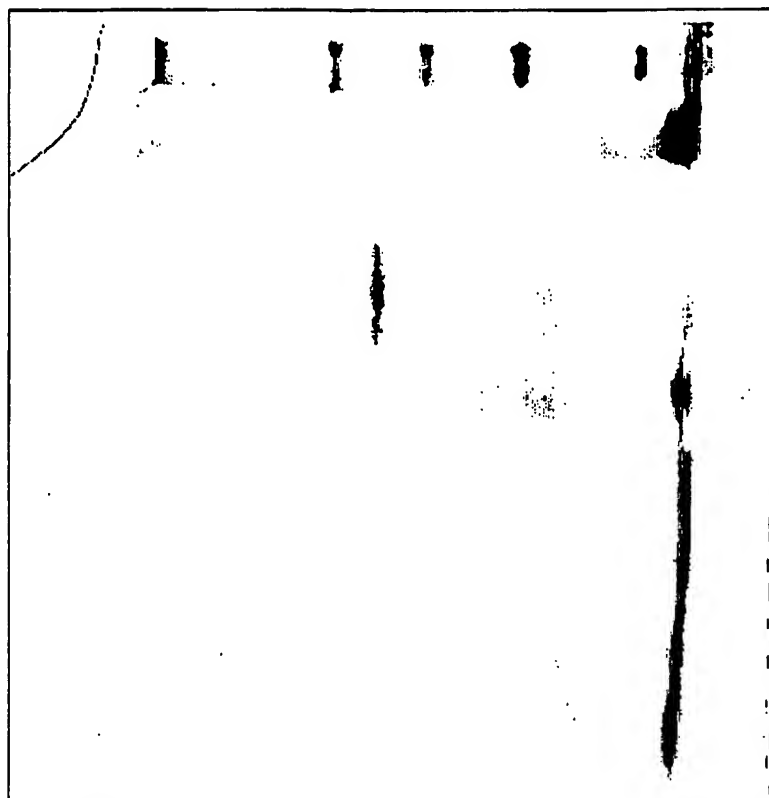


Fig. 6

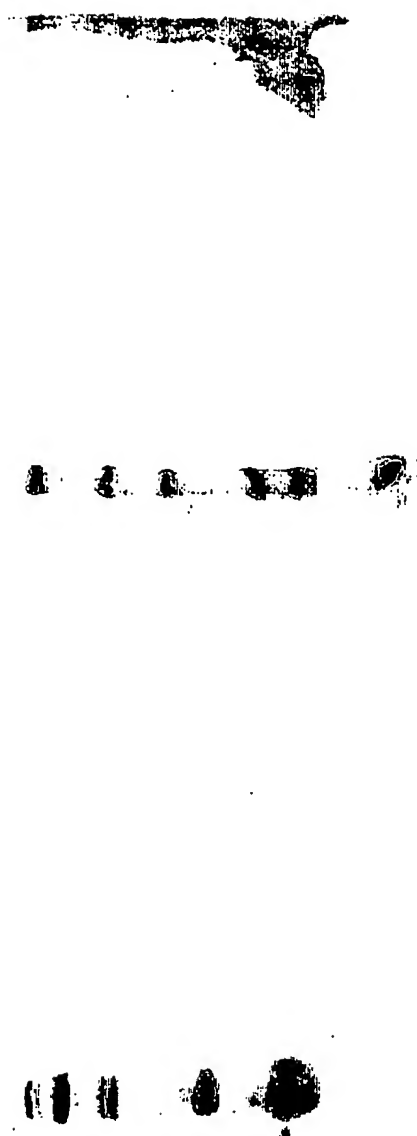


Fig. 5

6/6

As

α PrP mAb 3F4



B C C C A D S P P P B C C C A D S P P P C C C A D S P P P H S
PK - +

Fig. 7A

Fig. 7B

INTERNATIONAL SEARCH REPORT

ational Application No

PC/IL2004/000840

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/150948 A1 (SNOW ALAN D ET AL) 17 October 2002 (2002-10-17)	1,10-14
Y	the whole document paragraph '0026! claims 2,3,7	2-9, 15-20
X	US 6 287 789 B1 (KLOCK JOHN C) 11 September 2001 (2001-09-11)	21-26
Y	the whole document column 7, line 12 - line 40 column 8, line 26 - line 36 columns 9,10	2-9, 15-20
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	THUY L P ET AL: "A new quantitative assay for glycosaminoglycans" CLINICA CHIMICA ACTA, AMSTERDAM, NL, vol. 212, 1992, pages 17-26, XP002978288 ISSN: 0009-8981 the whole document page 18 -----	1-26

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